ISSN 0704-3716

Canadian Translation of Fisheries and Aquatic Sciences

No. 5456

Diarrhetic shellfish poison examination using monoclonal antibodies

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Original title: Monokuronaru Kotai o Riyo shita Gerisei Kaidoku no Kensa

In: BIO Industry 5(9): 39-46, 1988

Original language: Japanese

Available from:
Canada Institute for Scientific and Technical Information
National Research Council
Ottawa, Ontario, Canada K1A 0S2

1989

19 typescript pages

5456

Secretary of State / Secrétariat d'État

MULTILINGUAL SERVICES DIVISION – DIVISION DES SERVICES MULTILINGUES

90-00609/

TRANSLATION BUREAU BUREAU DES TRADUCTIONS

LIBRARY IDENTIFICATION – FICHE SIGNALÉTIQUE

Translated from - Traduction de	Into - En
Japanese	English

Author - Auteur

Taizo UDA and Takeshi YASUMOTO

Title in English or French - Titre anglais ou français

Diarrehtic Shellfish Poison Examination using Monoclonal Antibodies

Title in foreign language (Transliterate foreign characters)
Titre en langue étrangère (Transcrire en caractères romains)

Monokuronaru Kotai o Riyo shita Gerisei Kaidoku no Kensa

Reference in foreign language (Name of book or publication) in full, transliterate foreign characters.
Référence en langue étrangère (Nom du livre ou publication), au complet, transcrire en caractères romains.

Not given

Reference in English or French - Référence en anglais ou français

Bio Industry

Publisher - Éditeur	DATE OF PUBLICATION / DATE DE PUBLICATION			Page Numbers in original / Numéros des pages dans l'original
	Year / Année	Volume	Issue No. / Numéro	pp. 39–46
Place of Publication / Lieu de publication	1988	5	9	Number of typed pages / Nombre de pages dactylographiées: 18

Requesting Department / Ministère-Client: DFO

Translation Bureau No. / Notre dossier nº: 3511523

Branch or Division / Direction ou Division: Scientific Publications

Translator (Initials) / Traducteur (Initiales): PS

Person requesting / Demandé par: Heather Cameron

JUN / JUIN -1 1989

Your Number / Votre dossier nº:

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Date of Request / Date de la demande: May 9, 1989

Canada

SEC 5-111 (84-10)

Department of the Secretary of State of Canada | Secrétariat d'État du Canada

MULTILINGUAL SERVICES DIVISION – DIVISION DES SERVICES MULTILINGUES

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Client's No.–N° du client	Department – Ministère	Division/Branch – Division/Direction	City – Ville
	DFO	Scientific Publications	Ottawa
Bureau No.–N° du bureau	**Language – Langue**	**Translator (Initials) – Traducteur (Initiales)**	
3511523	Japanese	PS	JUN JUIN -1 1989

Diarrhetic Shellfish Poison Examination using Monoclonal Antibodies

by

Taizo UDA* and Takeshi YASUMOTO**

Summary

We manufactured monoclonal antibodies reacting specifically against both okada acid and dinophysis toxin-1, and developed an enzyme immunity measurement method using the same. By using this method, the diarrhetic shell poison examination is completed within 1 hour, including even the extraction of the samples. Compared to the erstwhile examinations based on bioassays and requiring 1 or 2 days, the time needed is much shortened. It is also possible to perform simultaneously the measurements of numerous samples.

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1. Introduction /39

Shell poisons may be widely divided into two types viz paralytic shell poison and diarrhetic shell poison. Together with paralytic shell intoxication, diarrhetic shell intoxication constitutes presently a grave problem. From the viewpoint of food health also, it is striking a heavy blow to the culture industry.

Some of the characteristics of diarrhetic shell poison are that the poisoning period may be long and that the poisoning occurs over a wide region. Recently, occurrences of intoxication and shell poisoning have been reported in Europe, South America and Australia.

The study of diarrhetic shell poison is quite recent. It started with food intoxication caused in 1976 by the ingestion of murasaki igai* from the Miyagi Prefecture. When investigating the cause of this food intoxication, Yasumoto et al[2] discovered in the midgut gland a fat-soluble poison killing mice, and called this poison fat-soluble shell poison[1)2)].

Later, it was called diarrhetic shellfish poisoning (DSP) as the main symptom of intoxication was diarrhea. The studies made by Yasumoto et al revealed that the poisoning causative species was the Dinophysis species of the dinophyceae[3)], and that the causative substances were the okada acid group, the pectenotoxin group and yesotoxin. Fig. 1 shows the chemical structure formulae of the causative poisons.

The toxicities are about 100 μg/kg with DTX_1, 250-770 μg/kg with the PTX group, and 100 μg/kg with the YTX group.

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*Translator's note: a type of mussel; Mytilus edulis LINNÉ

We manufactured monoclonal antibodies for the diarrhetic shellfish poison having recently become a problem[4], and developed an assay method enabling to detect traces of shellfish poison in a short period of time[5]. We will give a few explanations about the details of this development and about the prospects of future applications.

2. Present analytical method

In the analysis of foodstuffs, the problems are always the extraction method and the quantitative determination characteristics of the analysis. We will first explain simply the mainly employed bioassay /40
methods and the chromatography method after which we will explain the immunity measurement method employing the monoclonal antibodies.

2.1 Biomass assay method using mice

Fig. 1 shows the so-far followed official method[6].

After extracting the diarrhetic shellfish poison from the biosample, one performs the clean-up operation and prepares a test solution. This test solution is administered to mice peritoneally and one sees if the mice are dead or alive after 24 h.

The quantity of poison killing a 10-20 g mouse in 24 h is in the case of diarrhetic shellfish poison called 1 mouse-unit (MU). The actual mouse unit calculation is found through Table 1 from the smallest dose and from the greatest dilution rate bringing death within 24 h to at least 2 out of 3 mice constituting one group.

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2.2 Method based on chromatography

Let us explain simply the method of Lee, Yasumoto[7] using high speed liquid chromatography (HPLC).

Fig. 3 shows the procedure from the extraction of the midgut gland to the HPLC measurement. Fig. 4 and Fig. 5 show the chromatogram and calibration curve of OA and of DTX. /41

Some of the characteristics of this method are that the amount of contamined sample may be small, that the measurement sensitivity is as high as 100 ng/mL because of fluorescent labelling, and that the assay characteristics vs. OA and DTX_1 are good. The method is superior as the procedure is carried out by a team skilled in HPLC.

There are also reports of analysis methods for OA and DTX_1 based on gas chromatography[8]. However, as the molecules are large in both compounds, no analysis is possible as long as the hydroxyl groups have not been silyllated with a trimethylsilyllating agent which is a little inconvenient. /42

3. The preparation of monoclonal antibodies for diarrhetic shellfish poison

Recent progress in biotechnology has been remarkable, and cell fusion technology for the production of monoclonal antibodies is no exception. There is also a fair production of monoclonal antibodies for low molecular compounds having by themselves no immunogenicity.

Using as antigen okada acid which is one of the diarrhetic shellfish poisons, we made monoclonal antibodies by the method shown hereafter and established an enzyme immunity measurement method using the same. As it is expected to be a new detection method for diarrhetic shellfish poison, we will also explain in detail the conditions of the development.

3.1 Preparing the antigens

Following the method of Kitagawa et al[9] we combined okada acid (OA) separately with egg white albumin (OVA) and cow's serum albumin (BSA), and made conjugates to be used as antigens for immunity and as antigens for analysis. The method is briefly described in Fig. 6.

First, we dissolved OA in 95% dioxane, added N-hydroxysuccinimide and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide-metho-p-toluene-sulfonate, and reacted for 3 h at room temperature. After the reaction, we added water, extracted the OA activated esters with ethyl acetate, and reacted at 4°C for one night in pyridine against BSA or OVA. After the reaction, we performed twice dialysis against water and preserved under dry frozen conditions.

3.2 Cell fusion

We mixed the OA-OVA which is an immunogen thoroughly with Freund's complete adjuvant and administered 0.5 mL peritoneally in several places /43
to BALB/c mice (♀, 8 weeks old). We performed the immunity 4 times, excised the spleen 3 days after the final immunity and used it for cell fusion.

We performed cell fusion according to the method of Köehler and Milstein[10]. That is, we placed 5×10^7 mouse myeloma cells (X63-Ag8.653; 653) and 2.5×10^8 of the aforementioned mouse spleen lymphocytes in a 50 mL-capacity plastic conical centrifugal tube, and performed cell fusion by using 50% PEG 4000.

About 2 hours following the fusion, there appeared a well in which one noted cellular multiplication. We examined with ELISA prepared by coating an immunoplate with OA-BSA to see whether antibodies against OA

were contained in the culture supernatant.

We performed the cell cloning by the limiting dilution method. At such time, we used a 96 well culture plate for the cell incubation, and used BALB/c mouse thymus gland cells suspension as support cells (10^7/mL).

Obtaining thus 3 stocks recognized to react against either of OA and DTX_1 (OA-1, A-2, OA-3), we recloned and secured fusion cell stocks. As for the subclasses of the respective antibodies, OA-1 was IgA, OA-2 and OA-3 were IgG_1.

4. Cross reactivity of the monoclonal antibodies vs. diarrhetic shellfish poison

The monoclonal antibodies are made by using OA as antigen, and cross-reactions between OA and substances having an analogous chemical structure is unavoidable.

Table 2 shows with regard to the obtained 3 species of monoclonal antibodies the cross reactivities between the various diarrhetic shellfish poison compounds. In this Table, 1 represents the reactivities of the various monoclonal antibodies against OA. It gives on the basis of this standard the reactivity against the other compounds. As the Table shows, all monoclonal antibodies have practically the same cross reactivity to OA and DTX_1. Yet, there is virtually no reaction against PTX_1, YTX. The diarrhea causativity of PTX and YTX is weak or about imperceptible, so it should be possible to evaluate the diarrhea causativity by using monoclonal antibodies.

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5. The enzyme immunity measurement method using monoclonal antibodies

If we consider applying the enzyme immunity measurement method to practical fields, it goes without saying that the specificity to diarrhetic shellfish poison is high. The time for the total analysis which includes extraction must be short and the procedure should be simple.

So, we labelled monoclonal antibodies (OA-3) with western horseradish persioxidase and developed a measurement method based on the one-step assay. Fig. 7 shows the schema.

This method is called the one-step method ELISA.

5.1 Measurement based on the one-step method ELISA

For the analytical-use solidified antigens, we allowed a given amount of OA-BSA to be adsorbed in advance on transparent plastic (we used 96-hole or 8-hole immunoplates). Thereafter, in succession we put in the various wells the samples to be examined and the OA of known concentration which is the standard substance. At this time, one adds simultaneously the monoclonal antibodies labelled with the enzymes and allows for 10 min the antigen antibody reaction to take its course.

In this reaction, the enzyme-labelled monoclonal antibodies bring about a competing reaction between the solidified antigens and the free antigens inside the solution. Consequently and depending on the quantity of free antigens, the volume of enzyme-labelled antibodies combining with the solidified antigens varies also. That is, in the end, one may /44
measure the amount of antibodies combined with the solidified antigens. This is proportional to the volume of enzyme used for labelling. So, one performs the quantitative (or qualitative) analysis of OA by adding a

substrate, allowing the enzyme reaction to take place and measuring with a colorimeter (or by the eye) the degree of color development.

By this method, the antigen-antibody reaction takes 10 min and the substrate reaction 6 min so that the total time of the analysis is about 20 min. As for the measurement samples, one extracts the contaminated samples with 90% MeOH, and it is sufficient to analyze the filtrate following filtration so that the method is extremely simple.

Fig. 8 shows the calibration curve yielded by this method. As the Graph shows, the detection limit of OA is a few ng/mL and the measurement range 5 ng/300 ng/mL.

5.2 OA addition-recovery test

We extracted 5 g of midgut gland from commercial scallops, added 25 mL (5 volumes) of 90% methanol aqueous solution, homogenized for 1 min with a mixer and extracted the OA. After filtering this extract, we diluted the obtained filtrate twice with pure water and used this product as test solution.

By performing the OA addition-recovery test through the present method, the calculated values and the measured values of the OA volume added in the test were in virtual agreement as shown in Table 3.

5.3 Characteristics of the enzyme immunity measurement method /45

The characteristics of the afore-explained enzyme immunity measurement method are the following.

1. The preparation of the measurement samples is easy : extraction by 90% methanol is enough; there is no need for clean-up operation

2. The analysis time is barely 20 min : so far 24 h was needed

3. No mice are used for the analysis

4. The detection sensitivity is 0.0125 MU : 5 ppb with okada acid conversion prior to extraction

5. Numerous samples may be analyzed simultaneously.

As photograph 1 indicates, kits for enzyme immunity measurements of diarrhetic shellfish poison having these characteristics are already being sold.

6. Conclusion

We have explained mainly the enzyme immunity measurement method using monoclonal antibodies as a method of measuring diarrhetic shellfish poison which has in recent years become a problem.

At the present stage it is not possible to say that enough data have been gathered to be able to compare with the conventional bioassay method. There are however plans for collecting such study data and the particular advantages and disadvantages will be made clear.

At the present time the characteristic of the enzyme immunity measurement method is that it enables to treat numerous samples simultaneously. Through this method, one will obtain information about the state of poison distribution and extent of toxification, and so the method should be useful to determine the areas not affected by the poison or the periods in which the shellfish should be harvested. It also seems that bioassays based on mice might be useful in the case of dubious samples following screening based on the enzyme immunity measurement method.

Fig. 1 Chemical structural formula of diarrhetic shellfish poison

a okada acid
b yesotoxin (YTX)

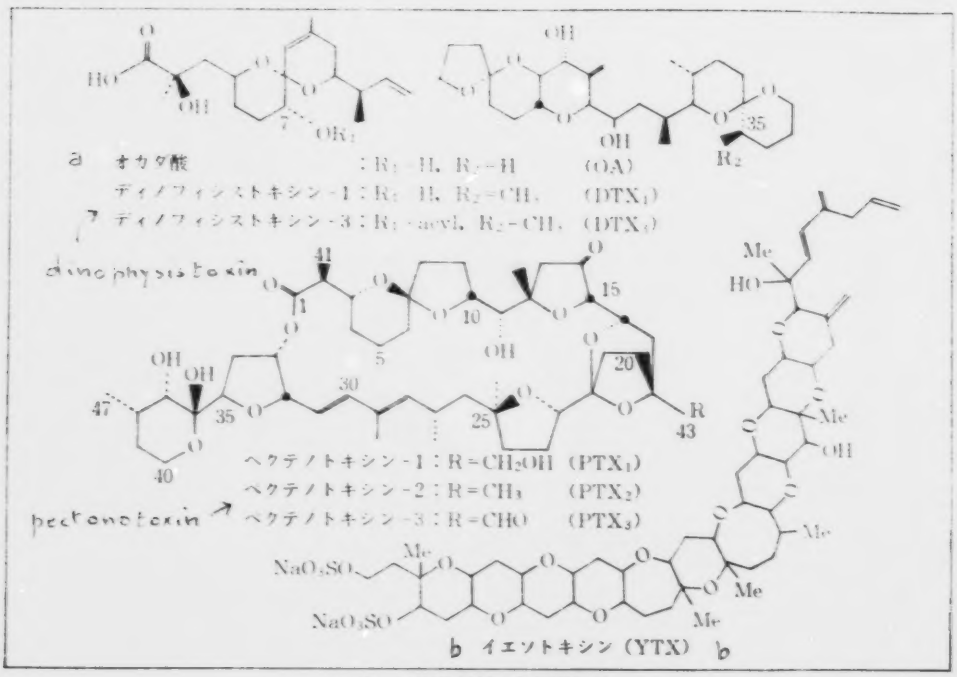

図1 下痢性貝毒の化学構造式

Fig. 2 Bioassay method using mice /40

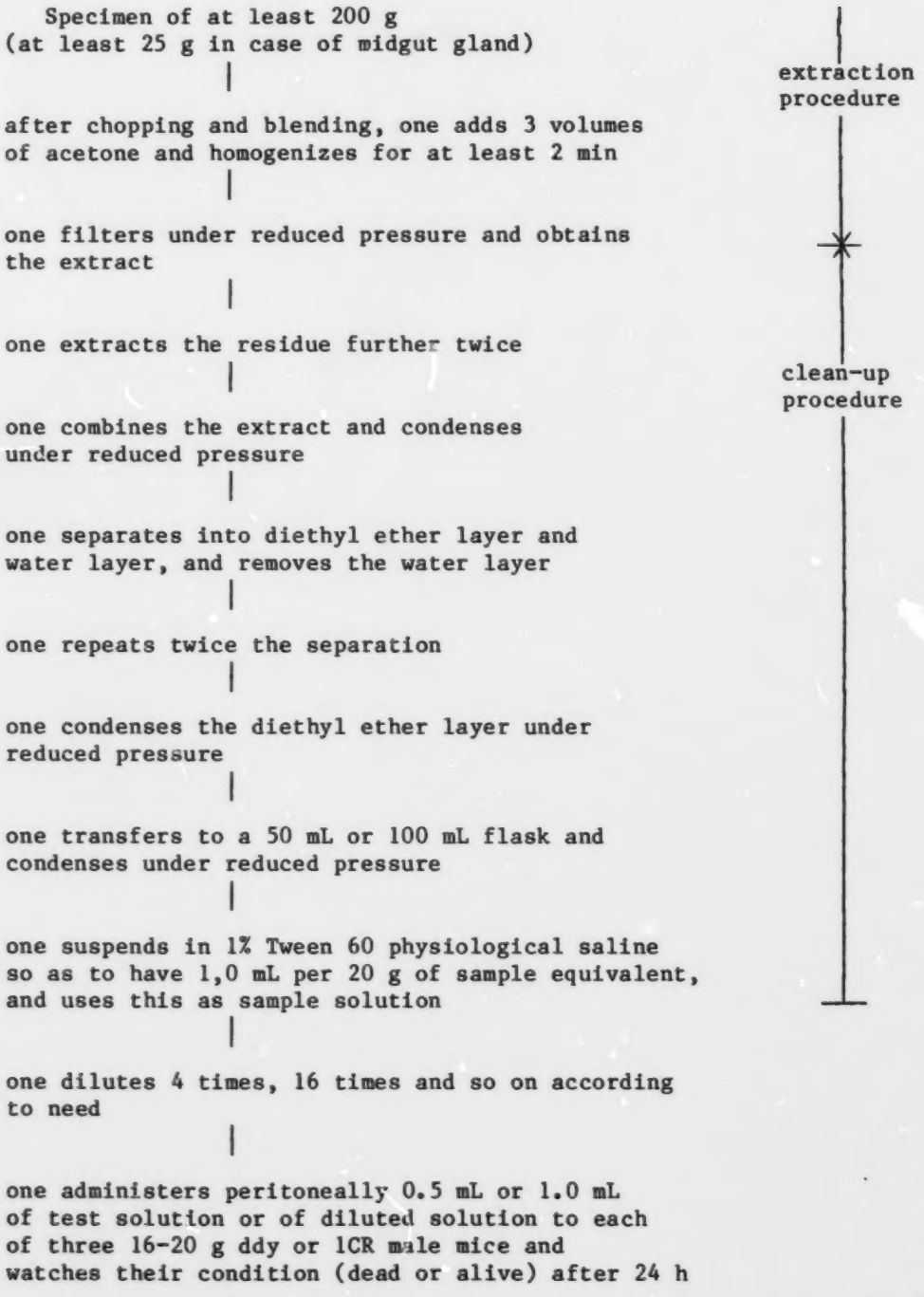
Specimen of at least 200 g
(at least 25 g in case of midgut gland)
after chopping and blending, one adds 3 volumes of acetone and homogenizes for at least 2 min
one filters under reduced pressure and obtains the extract
one extracts the residue further twice
one combines the extract and condenses under reduced pressure
one separates into diethyl ether layer and water layer, and removes the water layer
one repeats twice the separation
one condenses the diethyl ether layer under reduced pressure
one transfers to a 50 mL or 100 mL flask and condenses under reduced pressure
one suspends in 1% Tween 60 physiological saline so as to have 1,0 mL per 20 g of sample equivalent, and uses this as sample solution
one dilutes 4 times, 16 times and so on according to need
one administers peritoneally 0.5 mL or 1.0 mL of test solution or of diluted solution to each of three 16-20 g ddy or ICR male mice and watches their condition (dead or alive) after 24 h
extraction procedure
clean-up procedure

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Table 1 Relationship between dose and toxic strength

a test solution
b dose (mL)
c sample equivalent (g)
d toxic strength (MU/g)
e original solution
f diluted 4 times
g diluted 16 times
h the assay limit with this method is 0.05 MU and about 200 ppb with diarrhetic shellfish poison conversion

表1 投与量と毒力の関係

a 試験液	投与量 (mℓ) b	試料相当量 (g) c	毒力 (MU/g) d
e 原液	1.0	20	0.05
〃	0.5	10	0.1
f 4倍希釈液	1.0	5	0.2
〃	0.5	2.5	0.4
g 16倍希釈液	1.0	1.25	0.8
〃	0.5	0.625	1.6

h この方法による定量限界は0.05MUであり，下痢性貝毒換算で約200ppbである。

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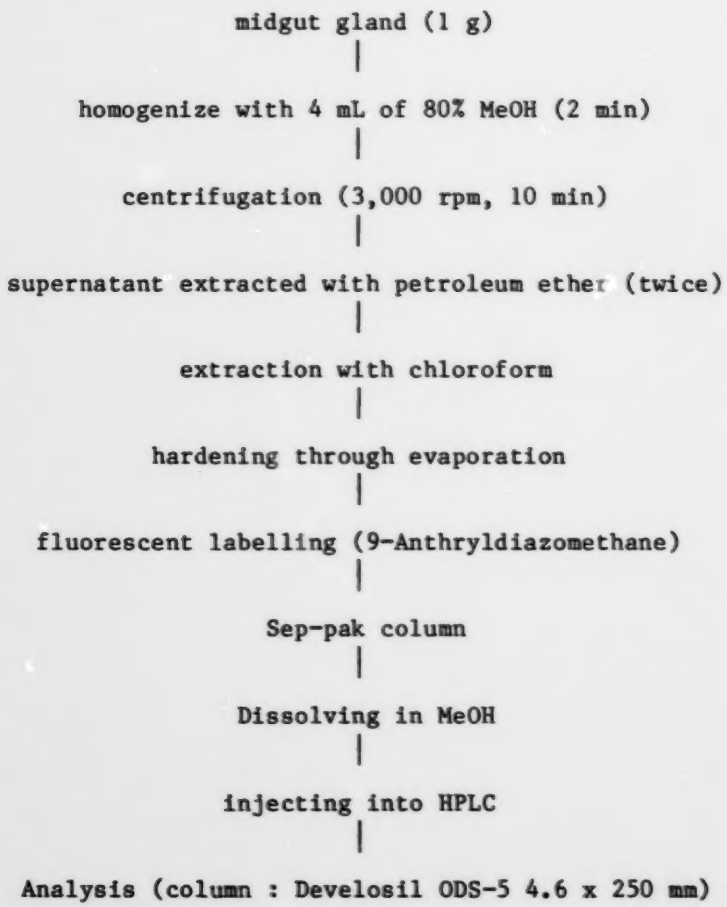
Fig. 3 Pretreatment needed for HPLC analysis


midgut gland (1 g)
homogenize with 4 mL of 80% MeOH (2 min)
centrifugation (3,000 rpm, 10 min)
supernatant extracted with petroleum ether (twice)
extraction with chloroform
hardening through evaporation
fluorescent labelling (9-Anthryldiazomethane)
Sep-pak column
Dissolving in MeOH
injecting into HPLC
Analysis (column : Develosil ODS-5 4.6 x 250 mm)

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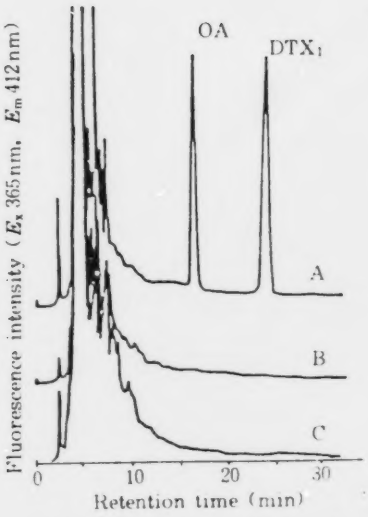

図4 蛍光 HPLC の分析チャート

Fig. 4 Fluorescent HPLC analysis chart

column : Develosil ODS (4.6 x 250 mm)
solvant : acetonitril - methanol - water (8:1:1)
current rate : 1.1 mL/min
detection : excitation wavelength 365 nm
fluorescence wavelength 412 nm

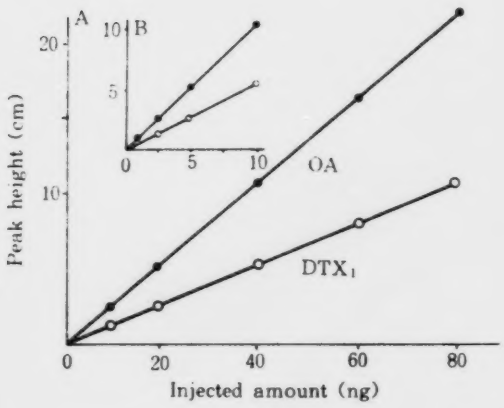

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Fig. 5 OA and DTX_1 calibration curves based on fluorescent HPLC analysis

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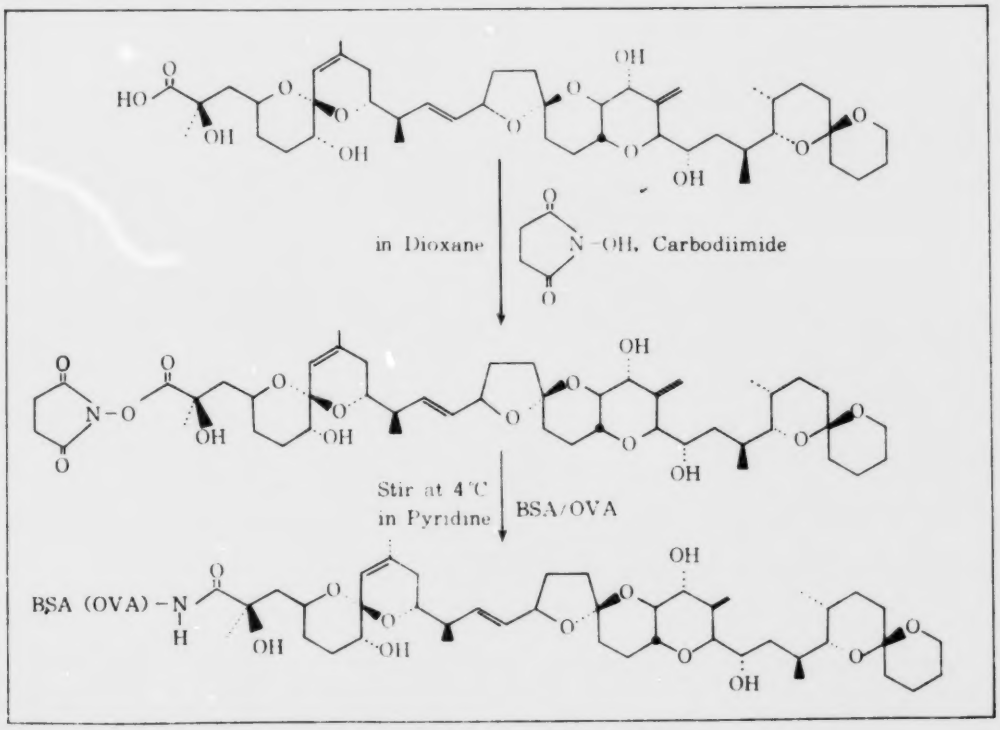

図6　免疫用および分析用抗原の作製法

Fig. 6 Method of preparing antigens for immunity and for analysis

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表2　各モノクローナル抗体の下痢性貝毒に対する交差反応性

a 貝毒	OA - 1	OA - 2	OA - 3
OA	1	1	1
DTX_1	1.07	0.87	0.74
PTX_1	< 0.0004	< 0.0003	< 0.0003
YTX	0.006	0.006	0.004

Table 2 Cross reactivities of the various monoclonal antibodies vs. the diarrhetic shellfish poisons

a shellfish poison

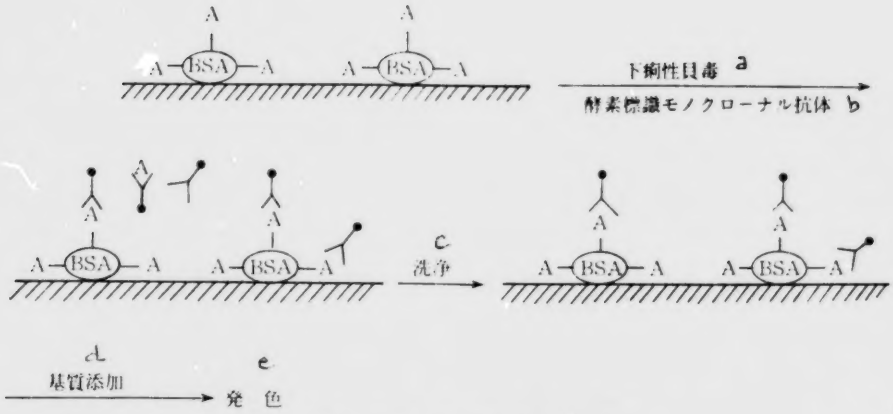

図7 下痢性貝毒の酵素免疫測定法

BSA：牛血清アルブミン　Y：POD 標識モノクローナル抗体　A：下痢性貝毒

Fig. 7 Enzyme immunity measurement method of diarrhetic shellfish poison

BSA; cow's serum albumin　　Y; POD labelled monoclonal antibody
A; diarrhetic shellfish poison

a diarrhetic shellfish poison
b enzyme-labelled monoclonal antibody
c washing
d substrate addition
e color development

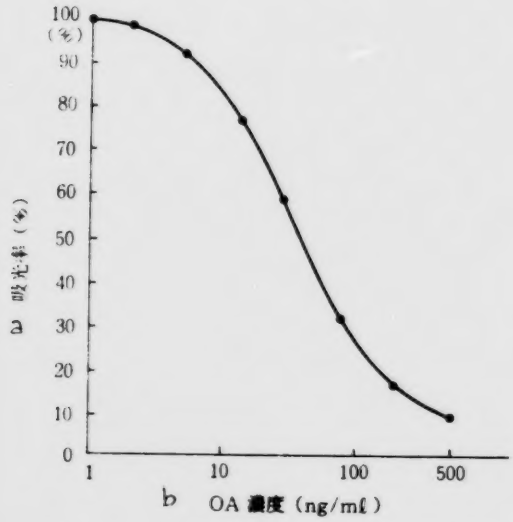

図8 ワンステップ ELISA によるオカダ酸の検量線

Fig. 8 One-step ELISA based okada acid calibration curve

a absorption rate (%)　　b OA concentration (ng/mL)

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表3 OA の添加回収試験

a OA 濃度 (ng/mℓ)		回収率 b
c 理論値	d 実測値	(%)
20	18	90
100	100	100
300	320	107

Table 3 OA addition-recovery test

a OA concentration
b recovery rate
c theoretical value
d measured value

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写真1 市販の酵素免疫法による下痢性貝毒検査キット

Photo 1 Commercial kit for diarrhetic shellfish poison examination based on enzyme immunity method

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